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Genomic structure, molecular characterization and functional analysis of Pekin duck interleukin-10

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ABSTRACT

Here we describe the cloning and expression of Pekin duck IL-10 (duIL-10) and a six exon-5 intron structure of an IL-10 gene. Two transcripts encoding duIL-10 with an alternatively spliced 3′UTR, and a transcript lacking exon 5 with a novel coding sequence for its C-terminus (duIL-10Δ*E5*) were isolated from splenocytes. The duIL-10 protein has an amino acid identity of 79% and 47% with chicken and human IL-10, respectively. The duck IL-10 gene shares a similar structure of the respective exons 1–5 with the IL-10 genes of other vertebrates but has an alternative exon. The duIL-10 3D structure by homology modeling was similar to that of the human IL-10 monomer, whereas the predicted duIL-10Δ*E5* protein lacks helix F. DuIL-10 and duIL-10Δ*E5* transcripts were most abundant in primary and secondary immune organs and lung. Recombinant duIL-10 suppressed duck IL-2 transcripts in mitogen-activated PBMCs. Our observation suggests evolutionary conservation of structure and function of the duIL-10 protein but the roles of the novel IL-10 splice variants in the regulation of duck immune responses and evolution of vertebrate immunity remain to be elucidated.

1. Introduction

Interleukin (IL)-10 is a homodimeric, four α-helix-bundle cytokine that exerts its biological effects through sequential binding to the class II receptor chains IL-10R1 (IL-10Rα) and IL-10R2 (IL-10Rβ), and activation of the JAK-STAT signaling pathway (Moore et al., 2001; Mosser and Zhang, 2008). IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), produced by murine T-helper 2 (Th2) cells, inhibiting the synthesis of pro-inflammatory cytokines by Th1 cells through its effects on antigen presenting cells (APCs) (Fiorentino et al., 1989, 1991). IL-10 has a central role in preventing inflammatory pathology and has emerged as a key immunoregulator during infections with viruses, bacteria, fungi, protozoa and helminthes (Couper et al., 2008; Ouyang et al., 2011; Wilson and Brooks, 2011). IL-10 has been identified across mammals and other vertebrates including avian (Rothwell et al., 2004) and amphibian species and bony fish (Zou et al., 2003; Savan et al., 2003; Zhang et al., 2005; Pinto et al., 2007; Seppola et al., 2008; Grayfer et al., 2011). All vertebrate IL-10 genes reported to date have a very similar genomic organization consisting of five exons and four introns.

IL-10 is produced by cells of the innate and adaptive immune system including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells and neutrophils, T cells and B cells (Wolk et al., 2002; Hedrich and Bream, 2010). In monocytes, DCs and macrophages IL-10 promotes inhibitory and tolerance-inducing functions of these cells (McBride et al., 2002; O'Garra and Murphy, 2009; Coquerelle and Moser, 2010). IL-10 also acts directly on T cells inhibiting T-helper responses and promoting the differentiation of IL-10-secreting regulatory T (Treg) cells (Maynard and Weaver, 2008). IL-10 functions as a dimer of two non-covalently linked monomers (Tan et al., 1993). Each chain consists of six amphipathic helices (A–F) that form a domain swapped, reciprocal dimer (Walter and Nagabhushan, 1995; Zdanov et al., 1995). Helices A, C, D, F′ and A′, C′, D′, F of each monomer adopt a classic four-alpha helix bundle. The structure of each IL-10 subunit is stabilized by two intra-molecular disulfide bridges (Windsor et al., 1993).

Both IL-10 receptor chains are comprised of tandem fibronectin-type-III (FBN-III) extracellular domains and each of their two subdomains consists of seven β-strands. A series of loops (L2–L6) that connect these β-strands within IL-10R1 interface with the surface formed by helix A, the AB loop, and helix F of the IL-10 homodimer (Zdanov et al., 1996; Josephson et al., 2001; Pletnev et al., 2005). Ligand binding to IL-10R1 leads to recruitment of IL-10R2, receptor heterodimerization and activation of the Janus

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kinases JAK1 and TYK2 which then leads to recruitment and activation of STAT3 and other latent transcription factors, depending on the type of cell (Finbloom and Winestock, 1995; Wehinger et al., 1996).

In this study we describe the cloning, sequence analysis and 3D modeling of the Pekin duck IL-10 ortholog (duIL-10), and a six exon-5 intron structure of the duck IL-10 gene that gives rise to two novel IL-10 splice variants. Furthermore, we provide an analysis of transcript expression in tissue and peripheral blood mononuclear cells (PBMCs) and a functional analysis of the expressed duIL-10 protein in a bioassay. Identification of duIL-10 provides the basis for further investigation of its role in immunity and inflammation in the duck, a non-mammalian vertebrate with significant relevance for human diseases, such as those caused by influenza A and hepatitis B.

2. Materials and methods

2.1. Animals, tissues and cell cultures

Pekin ducks (*Anas platyrhynchos*) used in this study were from a flock maintained at the University of Alberta. Animals were housed, maintained and handled according to the guidelines of the Health Sciences Laboratory Animal Services, University of Alberta. Tissues were from two-month-old Pekin ducks harvested at necropsy, snap-frozen in liquid nitrogen and stored at −70 °C. Splenocytes or PBMCs were isolated as previously described (Yao et al., 2010a). For analysis of IL-10 transcript expression upon mitogen activation, PBMCs were stimulated with phorbol myristic acid (PMA) (Sigma, final concentration 10 ng/ml) and ionomycin (Sigma, 1 μg/ml). Cells (1×10^7/ml) were harvested after 0, 2, 4, 8 and 24 h. To determine duIL-10 mRNA expression levels in tissue scrapings of frozen tissues were placed directly into Trizol (Invitrogen) and homogenized with a micropestle before RNA isolation (Yao et al., 2010a). To obtain the duIL-10 cDNA, total RNA was isolated from a 24-h splenocyte culture grown in the presence of concanavalin A (ConA) (Sigma, 1 μg/ml) and PMA (10 ng/ml) using Trizol reagent according to the manufacturer's protocol.

2.2. Isolation and sequencing of cDNA clones for duIL-10 and duIL-10ΔE5

First-strand cDNA synthesis was performed using 1 μg of RNA and Superscript reverse transcriptase (Invitrogen) and oligo dT followed by polymerase chain reaction using Taq polymerase (Invitrogen), according to the manufacturer's protocols. Primers FC and RC (Table 1) were selected based on the chicken IL-10 sequence (GenBank ID: NM_001004414) and duIL-10 amplicons were generated using 0.1 μg of cDNA, primers FC and RC in PCR buffer (Invitrogen) containing 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. PCR parameters were as follows: an initial step to denature at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, and at the end of cycles reactions were exposed to 72 °C for 5 min. Following agarose electrophoresis (1%), amplicons were isolated using a gel extraction kit (Qiagen) and cloned into the plasmid pCR4-TOPO (Invitrogen) according to the manufacturer's protocol. Clones bearing inserts were sequenced and BLAST comparison identified a clone, clone-352, as having an ORF showing homology with chicken IL-10 sequence. To determine the nucleotide sequences at the extreme 5′ and the extreme 3′ of the duIL-10 ORF (i.e. to which primers FC, RC bound), 5′ RACE and 3′ RACE were performed. Total RNA from duck splenocytes treated with ConA (1 μg/ml) and PMA (10 ng/ml) for 24 h was used as source material for 5′ RACE using commercially available kits from Invitrogen. For 5′ RACE, primer RIL10-1 was used for gene-specific cDNA synthesis and primers DuIL10-R and RIL10-2 were used as internal RACE primers (Table 1). Amplicons were purified, cloned and sequenced as described above. For 3′ RACE, primer UF4 and FIL10-5 were used for 1st and 2nd round PCR, respectively. Three 5′ RACE clones (5′-2, 5′-3, 5′-5) and three 3′ terminal clones (3′-1, 3′-2, 3′-3) were generated. The consensus sequence of 3′ RACE clones contains duIL-10 exon 4 sequence but has an in-frame deletion of exon 5 and a novel coding and noncoding sequence. To reveal putative alternately spliced transcripts generated from the 3′UTR of the predicted isoform (duIL-10Δ*E5*), primers UR3, UR4, UR5, UR6 and UR7 were designed based on the 3′UTR consensus sequence obtained from 3′ RACE and genomic sequence downstream of the 3′UTR identified in sequences (751025–751367 in scaffold 191) deposited by the Beijing Genomic Institute (BGI) at http://www.pre.ensembl.org/Anas_platyrhynchos/info/index (Table 1). The distance between these primers is around 50 bp, and the polyadenylation signal (TATAAA) is between UR6 and UR7. To reveal transcripts containing exon 1–5 with unspliced 3′UTR, RT–PCR was performed with primer F13 and primers UR8, UR9, UR10, UR11 and UR12, designed based on duIL-10 genomic sequence downstream the exon 5 coding region (751662–753996 in scaffold 191, BGI). UR11 contained the predicted "AATAGA" polyadenylation signal hexamer (Beaudoing et al., 2000). Two 3′ terminal clones (19-1, 19-2) generated had sequence homology.

Primers FF and FR1 amplify the entire ORF of duIL-10 and primers FF and FR2 amplify the entire ORF of duIL-10Δ*E5* (Table 1). High fidelity polymerase (*Pfx50*™ DNA Polymerase, Invitrogen) was then used according to the manufacturer's protocol (Invitrogen) to generate additional amplicons for cloning into pCR4-TOPO (Invitrogen) and verification of sequence of duIL-10 (clone 1–1, 1–4, 1–6) and duIL-10Δ*E5* (clone 2–16, 2–23, 2–24, 2–26). The consensus sequences derived from 14 clones for duIL-10Δ*E5*, duIL-10 (spliced 3′UTR) and duIL-10 (unspliced 3′UTR) transcripts are deposited in GenBank (GenBank ID: JN786939, GenBank ID: JN786940 and GenBank ID: JN786941).

Table 1
PCR primers and cycle conditions.

Name	Sequence(5′ → 3′)
FC	TTCTCCCCGCAGGAAGCCAGCCTG
RC	TCAAAGGTCCCCTTAAACTCATCCA
RIL10-1	TCGCCTTGTAGATGCCGTTC
DuIL10-F	TAAGGGGACCTTTGGCTGCCAGTC
DuIL10-R	ATGGTTTTGCTCCTCTTCTCGCAC
FIL10-1	AGATGATGCGGTTCTACATG
FIL10-5	ACTTTCAATCCCAAGACGATGA
RIL10-2	TTCAGGCTCAGCAGCAGGTT
F13	CGAGGAGATTAAAGACTACTTTCAATCCCAAGA
UF4	ATGAGGGGCTCCATCAAGGT
UR3	GAAGCGAGCAGGTCTGTCGGTAACTAATA
UR4	GGTCAGGTCCTCTGCCGCTGTATG
UR5	GCTGGGCTCTGGAAATGGCTGTG
UR6	CAGTGTTCTGGCTGGCAAGCAAAG
UR7	GACACTGGAGTGAAGTTTAGGCAGAAACT
UR8	TGATTTCTGAAGAAGTAGCAGGACTTGGTG
UR9	AATTCCTGACCTGAGCCCTAGCATAACT
UR10	CGGCAGAAGAATGCTGCTTTGGGT
UR11	TTTATGATTATTATTCTCAGTGCAAGGCCA
UR12	TCTATTAGAACCAAAAGCTTAATTCCTATCAC
UR13	TTTATTAGCAAAAATAAAAGGTTTCTTTTCAT
FF	ATGAGAACCTGCTGCGTGGCACTGCT
FR1	TCACTTCCTCCTCTTCATCAGCAAGTATTC
FR2	TTAGCTGAGAATGGAGCTGGTAGCTCCTG
FR3	TTCTCGAGACTTCCTCCTCTTCATCAGCAAGTA
FR4	TTCTCGAGAGCTGAGAATGGAGCTGGTAGCTCC

FC and RC are based on chicken IL-10, other primers are based on duck sequences. F: forward primer; R: reverse primer; FF: full forward (ORF); FR: full reverse (ORF). Denaturation – 94 °C, 3 min; amplification 1–35 cycles–94 °C, 30 s; 58 °C, 30 s; 72 °C, 60 s; final extension −72 °C, 5 min.